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☐ 1. Document ID: US 6312960 B1

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US-PAT-NO: 6312960
DOCUMENT-IDENTIFIER: US 6312960 B1

TITLE: Methods for fabricating an array for use in multiplexed biochemical analysis

DATE-ISSUED: November 6, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Balch; William J.	The Woodlands	TX		
Hogan; Michael E.	The Woodlands	TX		

US-CL-CURRENT: 436/518; 422/105, 422/112, 422/62, 422/63, 422/67, 422/68.1, 422/81, 435/286.1, 435/286.5, 435/286.6, 435/6, 436/43, 436/50, 436/524, 436/525, 436/527, 436/531

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☐ 2. Document ID: US 6083763 A

L7: Entry 2 of 2 File: USPT Jul 4, 2000

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DOCUMENT-IDENTIFIER: US 6083763 A

TITLE: Multiplexed molecular analysis apparatus and method

DATE-ISSUED: July 4, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Balch; William J.	Woodlands	TX		

US-CL-CURRENT: 436/518; 422/105, 422/112, 422/62, 422/63, 422/67, 422/68.1, 422/81, 435/286.1, 435/286.5, 435/286.6, 435/6, 436/43, 436/50, 436/524, 436/525, 436/527, 436/531

Full | Title | Citation | Front | Review | Classification | Date | Reference KWMC | Draw. Desc | Image

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L7: Entry 2 of 2 File: USPT Jul 4, 2000

DOCUMENT-IDENTIFIER: US 6083763 A
TITLE: Multiplexed molecular analysis apparatus and method

BSPR:
It is very desirable to rapidly detect and quantify one or more molecular structures in a sample. The molecular structures typically comprise ligands, such as antibodies and anti-antibodies. Ligands are molecules which are recognized by a particular receptor. Ligands may include, without limitation, agonists and antagonists for cell membrane receptors, toxins, venoms, oligosaccharides, proteins, bacteria and monoclonal antibodies. For example, DNA or RNA sequence analysis is very useful in genetic and infectious disease diagnosis, toxicology testing, genetic research, agriculture and pharmaceutical development. Likewise, cell and antibody detection is important in numerous disease diagnostics.

BSPR:
In particular, nucleic acid-based analyses often require sequence identification and/or analysis such as in vitro diagnostic assays and methods development, high throughput screening of natural products for biological activity, and rapid screening of perishable items such as donated blood, tissues, or food products for a wide array of pathogens. In all of these cases there are fundamental constraints to the analysis, e.g., limited sample, time, or often both.

BSPR:
In these fields of use, a balance must be achieved between accuracy, speed, and sensitivity in the context of the constraints mentioned earlier. Most existing methodologies are generally not multiplexed. That is, optimization of analysis conditions and interpretation of results are performed in simplified single determination assays. However, this can be problematic if a definitive diagnosis is required since nucleic acid hybridization techniques require prior knowledge of the pathogen to be screened. If symptoms are ambiguous, or indicative of any number of different disease organisms, an assay that would screen for numerous possible causative agents would be highly desirable. Moreover, if symptoms are complex, possibly caused by multiple pathogens, an assay that functioned as a "decision tree" which indicated with increasing specificity the organism involved, would be of high diagnostic value.

BSPR:
Blotting techniques, such as those used in Southern and Northern analyses, have been used extensively as the primary method of detection for clinically relevant nucleic acids. The samples are prepared quickly to protect them from endogenous nucleases and then subjected to a restriction enzyme digest or polymerase chain reaction (PCR) analysis to obtain nucleic acid fragments suitable for the assay. Separation by size is carried out using gel electrophoresis. The denatured fragments are then made available for hybridization to labeled probes by blotting onto a membrane that binds the target nucleic acid. To identify multiple fragments, probes are applied sequentially with appropriate washing and hybridization steps. This can lead to a loss of signal and an increase in background due to non-specific binding. While blotting techniques are sensitive and inexpensive, they are labor intensive and dependent on the skill of the technician. They also do not allow for a high degree of multiplexing due to the problems associated with sequential applications of different probes.

BSPR:
Microplate assays have been developed to exploit binding assays, e.g., an ELISA assay, receptor binding and nucleic acid probe hybridization techniques. Typically, with one microplate, e.g., micro-well titer plate, only one reading per well can be taken, e.g., by light emission analysis. These assays function in either one of two ways: (1) hybridization in solution; or (2) hybridization to a surface bound molecule. In the latter case, only a single clement is immobilized per well. This, of course, limits the amount of information that can be determined per unit of sample. Practical considerations, such as sample size, labor costs, and analysis time, place limits on the use of microplates in multiplex analyses. With only a single analysis, reaction, or determination per well, a multiple pathogen screen with the appropriate controls would consume a significant portion of a typical 96 well format microplate. In the case where strain determination is to be made, multiple plates must be

BSPR:
Various strategies have been implemented to enhance the accuracy of these probe-based hybridization assays. One strategy deals with the problems of maintaining selectivity with assays that have many nucleic acid probes with varying GC content. Stringency conditions used to eliminate single base mismatched cross reactants to GC rich probes will strip AT rich probes of their perfect match. Strategies to combat this problem range from using electrical fields at individually addressable probe sites for stringency control to providing separate micro-volume reaction chambers so that separate wash conditions can be maintained. This latter example would be analogous to a miniaturized microplate. Other systems use enzymes as "proof readers" to allow for discrimination against mismatches while using less stringent conditions.

BSPR:
Although the above discussion addresses the problem of mismatches, nucleic acid hybridization is subject to other errors as well. False negatives pose a significant problem and are often caused by the following conditions:

BSPR:
Another use of the microplate based arrays is for the creation of hierarchical arrays for complex analyses. In this format, multiple arrays operate in parallel to provide an answer to a complex assay. The example of the diagnostic assay provided in the Background section illustrates some of the parameters which should be considered in order to provide an accurate result. For any specific analysis, a set of probe elements must be chosen. The selected probe elements should be able to selectively associate with defined targets without significant cross association to other macromolecules expected from either the patient or other organisms commonly associated with a specific sample type. Controls must be designed to prevent false positive or negative results from the sources outlined in the Background section. Once this is done, a combinatorial process can be used to identify the optimal association and selectivity conditions for the defined analysis. For nucleic acid applications, these conditions are highly dependent on the capture probe length and composition, target base composition, and sample matrix. The number of arrays to be used depends on a number of different factors, e.g., the controls to be implemented and the differences in base composition of the capture probes. Ultimately, a set of integrated chemical devices emerge that can rapidly, efficiently, and accurately provide an answer for the molecular analysis of interest.

BSPR:
Another use of the hierarchical arrays and the reaction vessel based arrays would be for screening, samples for a broad range of possible targets. In one case, a diagnostic test is performed to search for the cause of a defined set of symptoms. In most cases this narrows the range of possible organisms to a small number. Conversely, to screen donated blood or tissue for a broader range of disease organisms, a decision tree approach could be employed. Here an initial array or set of arrays could be chosen to screen for broader classes of pathogens using probes for highly conserved nucleic acid regions. Results from this would indicate which additional array sets within the microplate to sample

next, moving to greater and greater specificity. If enough sample is available, as might be the case with donated blood or tissue, all of the decision tree elements could be interrogated simultaneously. If sample quantity is limiting, the approach could be directed in a serial fashion.

BSPR:
Assay development for any multiplex analysis is time consuming. The microplate based arrays as described herein can be used to speed the process for capture probe/target binding or hybridization. A defined array can be deposited into each well of a microplate and then the association reactions are carried out using "gradients" of conditions that vary in two dimensions. For example, consider a 96 well microplate containing nucleic acids arranged in 8 rows by 12 columns. In one step of the optimization, the effects of pH on various substrate compositions might be examined to see how this affects hybridization specificity. Twelve different pH's, one for each column, and 8 different surface chemistries, one for each row could be used under otherwise identical hybridization conditions to measure the effects on hybridization for each capture probe/target element in the array. This type of analysis will become essential as array technology becomes widely used and is amenable to any receptor/ligand binding type experiment.

BSPU:
1) Unavailability of the binding domain often caused by intra-strand folding in the target or probe molecule, protein binding, cross reactant DNA/RNA competitive binding, or degradation of target molecule.

DEPR:
By "capture probe, probe molecules or probes" is meant the molecules which are deposited as biosites onto the reaction substrate for interrogating the target molecules. Probes are meant to include nucleic acids, DNA, RNA, receptors, ligands, antibodies, anti-antibodies, antigens, proteins, and also small chemical compounds such as drugs, haptens, or peptides.

DEPR:
The term "oligonucleotide probe arrays" refers to probe arrays wherein the probes are constructed of nucleic acids.

DEPR:
By "ligands" is meant molecules which are recognized by a particular receptor. "Ligands" may include, without limitation, agonists and antagonists for cell membrane receptors, toxins, venoms, oligosaccharides, proteins, bacteria and monoclonal antibodies.

DEPR:
By "biosite" is meant the biological molecules or capture probes that are deposited on the top surface of the reaction substrate, or base material. Under appropriate conditions, an association or hybridization can occur between the capture probe and a target molecule. The component strands of the biological molecule form the biosite since there is the potential of a reaction occurring between each component strand of the biological molecule and the target molecule. For example, each reaction chamber can contain at least one biosite. The maximum number of biosites per reaction chamber will depend on the size of the reaction vessel and on the practical optical resolution of the accompanying detector/imager. For example, an array of 16 (4.times.4 array) biosites may be deposited on the hybridization substrate or base material that eventually forms the bottom of the entire reaction vessel. Each biosite comprises a circle of approximately 25-200 microns (.mu.m) in diameter. Thus, for a 16 biosite array, each of the 16.times.200 .mu.m diameter area contains a uniform field of probes attached to the hybridization substrate (base material) in a concentration which is highly dependent on the probe size and the well size. Each 25-200 .mu.m diameter area can contain millions of probe molecules. Also, each of the 16 different biosites (probe sites) can contain one type of probe. Thus, 16 different probe types can be assayed in an array containing 16 biosites (4.times.4 array) per reaction chamber. As another example, four separate 10.times.10 arrays (400 biosites) can be generated to fit into one well of a 96

well microtiter plate with sufficient spacing between each of the 400 biosites. For this 10.times.10 format, 400 hybridization experiments are possible within a single reaction chamber corresponding to 38,400 (96.times.400) assays/hybridization that can be performed nearly simultaneously.

DEPR:
STEP 2. Self Assembling Arrays--Universal Arrays. Creating and constructing self assembling probe arrays or universal arrays enables on-line configuration of the biosites wherein an unvarying probe array (capture probes) is activated by binding to a cognate set of adapters (target probes) to yield a modified probe array which is specifically configured for analysis of a target or target mixture. For this invention, "cognate" is defined for nucleic acids as a sequence which is complementary by the means of Watson-Crick pairing to another sequence.

DEPR:
The lid of the storage vessel holder can be attached to a Z-axis motion control device to allow for automated changes of the biosite solutions contained in the microplates which may be delivered by a robotic arm. This is useful for printing sets of arrays containing large numbers of solutions, such as small molecule libraries used in drug discovery.

DEPR:
The embodiment depicted employs 190 .mu.m OD capillaries, which are threaded through an attachment site at the top of the printing fixture. The tubes extend down from the attachment site through an area that allows for the capillaries to flex during printing. Below the flex region the capillaries are threaded through an array template or a set of fused silica sleeves held in a grid pattern by the aluminum holder assemblies. The capillary sleeves/array template constitute an important innovation. The array templates/capillary sleeves also allow the capillary tubing to travel smoothly and independently with respect to each other in the vertical axis during printing.

DEPR:
In photolithographic microarray synthesis, a series of masks are sequentially applied to build the nucleic acid probes a base at a time. An array of oligonucleotide probes each 12 bases long would require 48 masks (12 nucleotide positions.times.4 bases). This process takes approximately 16 hours to complete a wafer containing 48 microarrays.

DEPR:
The enclosed printing solution storage vessel is purged with an inert gas during the priming step of the printing process, which also serves to maintain an inert environment for the probe solutions. Contamination of the probe solutions is minimized because of the single direction of flow through the capillaries. The printing end does not dip into the reservoir after every print cycle as in the load and dispense techniques. This is important with contact printing where the depositors touch the surface of the chip or slide that will contain the microarray. These surfaces are chemically treated to interact or bind to the probe solutions. Residual reactive chemicals, or even dust and dirt could be introduced into the probe solution supply chambers with load and dispense systems. Often, the solution to be printed is available in limited quantity or is very expensive. This is often the case in pharmaceutical drug discover applications where small molecule libraries, containing hundreds of thousands of unique chemical structures that have been synthesized or collected and purified from natural sources, are used in high throughput screens of as many potential disease targets as possible. These libraries must be used as efficiently as possible. The amount of fluid that is required for each printing system varies depending on the design. Most require a minimum of 100 microliters (.mu.L) and are able to print less than 1,000 slides, with a significant amount of solution lost to washing between print cycles. The capillary array printer requires only 3 .mu.L with less than 1 .mu.L used for the initial priming. This volume of printing solution is sufficient to print between 20,000 to 30,000 microarrays with each capillary dispensing 50 to 100 pL per array. Load and dispense systems deliver anywhere from 800 pL to several nL per array.

DEPR:
The flexible fused silica tubing (or other suitable material such as glass, Teflon or other relatively inert plastic or rubber, or thin, flexible metal, such as stainless steel) originating at the printing storage vessel, pass through a series of arraying templates or sleeves that are held at specific locations in the print head. An attachment site holds the capillaries in a fixed position that does not generally allow horizontal or vertical movement. The capillaries extend down from this anchor point through an open area ("flexation zone") and into a set of array templates or sleeves. These lower array templates or sleeves serve to hold the printing capillaries in a geometry that matches the microarray to be printed. The array templates limit the lateral movement of the printing capillaries to preserve the correct printing pattern, while allowing unrestricted vertical movement of each printing capillary independently of each other. This feature allows the print head to print on slightly irregular or uneven surfaces. The print head moves downward to contact the substrate that is to receive the probe solutions, after the initial contact, the downward movement continues (the distance depends on the surface, from 100 .mu.m to a few mm) to ensure that all of the printing capillaries contact the surface. The flexation zone positioned between the attachment site (that is holding the capillaries fixed) and the array templates or sleeves allows each capillary to bend so as to accommodate the "overdrive" of the print head. When the print head moves up away from the substrate, the printing capillaries straighten out again.

DEPR:
In an alternative embodiment, the capillary tubes may be essentially rigid tubes (e.g., stainless steel) mounted in flexible or movable fashion at the attachment site, and slidably held by an array template. In this embodiment, the plurality of capillary tubes can be pressed against a reaction substrate and "even up" at their distal ends by moving lengthwise through the array template, thus accommodating uneven deposition surfaces.

DEPR:
The surface bound universal capture probes are arranged in an array of biosites attached to a solid support. Each biosite consists of a multitude of specific molecules distinct in function or composition from those found in every other biosite in the array. These capture probes are designed to have a specific composition or sequence to provide rapid and efficient binding to the capture domain of the target probes. The specific composition is also chosen to minimize cross association between capture probes and their specific target probes.

DEPR:
Specifically for a nucleic acid capture probe the surface bound capture array should be designed for optimum length, base composition, sequence, chemistry, and dissimilarity between probes.

DEPR:
The length of the nucleic acid capture probe should be in the range of 2-30 bases and preferably in the range of 5-25 bases. More preferably, the length ranges from about 10-20 bases and most preferably is at or about 16 bases in length to allow for sufficient dissimilarity among capture probes. Length is also adjusted in this range to increase target probe binding affinity so that capture probe arrays can be activated by addition of target probe mixtures as dilute as 10.sup.-9 M. This allows target probes to be synthesized in small scale and inexpensively. Also, length is adjusted to this range to reduce the rate of target probe dissociation from capture probe arrays. This allows the activated capture probe arrays to be washed thoroughly to remove unbound target probes, without dissociation of specifically bound target probes from the surface. With capture probes in such a size range, the complex formed by and between the target probe and capture probe interaction is stable throughout subsequent air drying, and can be stored indefinitely with refrigeration.

DEPR:
A preferred percentage base composition for capture probe array sets is in the range of at or around 30-40% G, 30-40% C, 10-20% A, 10-20% T. Relatively G+C

rich capture probes are desirable such that the thermodynamic stability of the resulting capture/target probe pairing will be high, thus allowing for surface activation at low added target probe concentrations (e.g., in the range of 10.sup.-9 M). Nearest neighbor frequency in the capture probe set should minimize G-G or C-C nearest neighbors. This criterion minimizes the possibility of side reactions, mediated via G-quartet formation during capture probe attachment to the surface, or during the capture probe-target probe binding step.

DEPR:
The capture probe should be linked to a solid support. This can be done by coupling the probe by its 3' or 5' terminus. Attachment can be obtained via synthesis of the capture probe as a 3' or 5' biotinylated derivative, or as a 3'/5' amine modified derivative, a 3'/5' carboxylated derivative, a 3'/5' thiol derivative, or as a chemical equivalent. Such end-modified capture probes are chemically linked to an underlying microtiter substrate, via interaction with a streptaviden film (for biotin), coupling to surface carboxylic acids or epoxide groups or alkyl halides or isothiocyanates (for amines) to epoxides or alkyl halides (for thiols) or to surface amines (for carboxylic acids). Other attachment chemistries readily known to those skilled in the art can be substituted without altering general performance characteristics of the capture probe arrays. Capture probe arrays can be fabricated by such chemistries using either robotic or micro ink jet technology.

DEPR:
A target probe set is designed and constructed to bind to the capture probe set in a specific manner, i.e., each target probe element binds to only one element of the capture probe set. Thus, a mixture of target probes can be administered to a capture probe array formed on the bottom of a microtiter well, or equivalent surface. For the nucleic acid embodiment of the Universal Array, subsequent to binding, the target probe set will partition itself among capture probe set members via Watson-Crick base pairing, thereby delivering a unique binding domain (cognate to analyte) to each site in the probe array.

DEPR:
There are two general methods that can be employed by the end-user to synthesize customized nucleic acid-based bifunctional target probes. The simplest and most direct method is to synthesize a single oligonucleotide that contains the two domains (capture and analyte) separated by a linker region using a standard automated DNA synthesizer. As a class, the bifunctional target probes for a nucleic acid embodiment possess a structural domain cognate to the capture probe which is the Watson-Crick complement to one element of the capture probe set. Its length and base sequence is thus defined by that of the capture probe via standard rules of antiparallel Watson-Crick duplex formation. In addition, the target probe also contains one of the following structural domains:

DEPR:
a. Cognate to a Small Segment of a Solution State Nucleic Acid Target Analyte

DEPR:
This is the component of the target probe which is complementary via Watson-Crick pairing to the solution state target nucleic acid to be analyzed. In general, its sequence has no correlation to that of the domain which is cognate to the capture probe. However, several general design criteria should be met.

DEPR:
This domain essentially creates nucleic acid amplification primers with tails complementary to capture probe sites in a Universal Array. After amplification, the resulting amplicon sets can be directly hybridized to the capture probe array and analyzed as described below.

DEPR:
Another method of synthesizing bifunctional DNA target probes consists of individually and separately synthesizing analyte and capture sequence oligos

that are chemically altered to incorporate a reactive functionality which will allow subsequent chemical linkage of the two domains into a single bifunctional molecule. In general, the 5' or 3' terminus of each oligo is chemically altered to facilitate condensation of the two sequences in a head to tail or tail to tail manner. A number of methods are known to those skilled in the art of nucleic acid synthesis that generate a variety of suitable functionalities for condensation of the two oligos. Preferred functionalities include carboxyl groups, phosphate groups, amino groups, thiol groups, and hydroxyl groups. Further, chemical activation of these functionalities with homo- or heterobifunctional activating reagents allows for condensation of the activated oligo with the second functionalized oligo sequence. Some examples of the various functionalities and activating reagents that lead to condensation are listed below:

DEPR:
A specific example of the target probe domains that are cognate to the capture probe set of the Universal Array and can be modified to allow for direct binding to a specifically modified probe, nucleic acid or other molecule capable of selective binding to the analyte of interest is illustrated in FIG. 5b. FIG. 5b is a diagram showing direct binding for a target probe. As shown in FIG. 5b, the target probe is constructed from two parts; the first is a presynthesized probe (TP1) complementary to a capture probe which has a linkage element for attaching the second target complex (TP2). Such embodiment yields a high degree of simplicity for the customer since the first target component can be offered in a ready-to-use format.

DEPR:
In some instances, a chemical linker may be needed to separate the two nucleic acid domains of the target probe, to minimize stearic interaction between the target probe and the solution state nucleic acid analyte. This linker may be constructed from nucleic acid building blocks. For example, the sequence T.sub.n (where n=1-5) is preferred because stretches of T are readily synthesized and minimize the likelihood of sequence dependent interactions with capture probe, other target probe domains, or the solution phase nucleic acid analyte.

DEPR:
4. Non-Nucleic Acid Embodiments

DEPR:
Small molecule Universal Arrays can be employed for rapid, high throughput drug screening. In this format, surface bound capture probes consist of small haptens or molecules arranged in separated biosites attached to a solid support. Each biosite consists of specifically-addressable, covalently immobilized small molecules such as haptens, drugs and peptides. These organic capture molecules are designed to have a high affinity association with a bispecific ligand. These ligands contains both a domain cognate to the small immobilized organic molecule (capture probe) and cognate to the analyte of interest. The domain cognate to the analyte can associate either directly to this target or to a label on the analyte.

DEPR:
Analytes can include, but are not limited to, dsDNA, ssDNA, total RNA, mRNA, rRNA, peptides, antibodies, proteins, organic enzyme substrates, drugs, pesticides, insecticides and small organic molecules.

DEPR:
Conversely, the format for a small molecule Universal Array can be inverted so that the macromolecular ligand becomes the capture probe. Thus, a Universal Array (Macromolecular Universal Array) may contain large macromolecules such as, without limitation, antibodies, proteins, polysaccachrides, peptides, or receptors as the immobilized capture probe. In turn, unique small molecule tags having a specific, high affinity association for the macromolecular biosites are covalently attached to various probes cognate to the analyte. These labeled probes now represent the bispecific component cognate to both the capture macromolecule and the target analyte. Some representative examples of small

molecules (haptens or drugs) are listed in Table 1 below. This is only a partial list of commercially available antibodies to haptens, steroid hormones and other small molecule drugs. Examples of these bispecific, small molecule-labeled macromolecules include antibodies, receptors, peptides, oligonucleotides, dsDNA, ssDNA, RNA, polysaccharides, streptavidin, or lectins. A partial list of 48 representative compounds for which specific antibodies are available include: fluorescein; dinitrophenol; amphetamine; barbiturate; acetaminophen; acetohexamide; desipramine; lidocaine; digitoxin; chloroquinine; quinine; ritalin; phenobarbital; phenytoin; fentanyl; phencyclidine; methamphetamine; metaniphrine; digoxin; penicillin; tetrahydrocannibinol; tobramycin; nitrazepam; morphine; Texas Red; TRITC; primaquine; progesterone; bendazac; carbamazepine; estradiol; theophylline; methadone; methotrexate; aldosterone; norethisterone; salicylate; warfarin; cortisol; testosterone; nortrptyline; propanolol; estrone; androstenedione; digoxigenin; biotin; thyroxine; and triiodothyronine.

DEPR:
The general concept of Universal Arrays, whether they be DNA-based, small molecule-based, or protein-based allows for great versatility and end-user friendliness. The various configurations described allow for highly parallel, simultaneous, multiplexed, high throughput screening and analysis of a wide variety of analyte mixtures.

DEPR:
Labeling can be achieved by one of the many different methods known to those skilled in the art. In general, labeling and detection of nucleic acid hybrids may be divided into two general types: direct and indirect. Direct methods employ either covalent attachment or direct enzymatic incorporation of the signal generating moiety (e.g., isotope, fluorophore, or enzyme) to the DNA probe. Indirect labeling uses a hapten (e.g., biotin or digoxigenin) introduced into the nucleic acid probe (either chemically or enzymatically), followed by detection of the hapten with a secondary reagent such as streptavidin or antibody conjugated to a signal generating moiety (e.g., fluorophore or signal generating enzymes such as alkaline phosphatase or horseradish peroxidase).

DEPR:
Fluorescent labeling is suitable for this invention for several reasons. First, potentially hazardous substances such as radioisotopes are avoided. Furthermore, the fluorescent labeling procedures are simpler than chemiluminescent methods since the latter requires enzymatic reactions and detection in the solution state. Finally, the fluorescent labeling approach can be modified to achieve the highest signal-to-nose ratio SNR among the safest labeling techniques by utilizing secondary linker chemistries that enable the attachment of hundreds of fluorescent dye molecules per target molecule.

DEPR:
Electrochemiluminescence or electrical chemiluminescence (ECL) labeling, e.g., ruthenium (Ru) does not require a wash step to remove excess target from the solution and is highly sensitive. Briefly, for electrochemiluminescence as a method of detection, the internal surface of the reaction chamber is coated with a conductive material, e.g., gold, and the biosite is attached to this conductive surface (See FIG. 6). FIG. 6 is a diagram showing ECL implementation in reaction vessel with proximal CCD imaging. Using one microtiter well (of a 96 microtiter well plate) as a reaction chamber, the biosites are deposited onto the internal circumference of the microtiter well by one of several methods as described above (ink-jet, capillary, or photolithography/capillary).

DEPR:
The specific ECL label, e.g., Ru, is attached to the target molecule by the conventional means. The labeled target is added to the hybridization solution and once hybridization occurs between the Ru labeled target and biosite, e.g., after sufficient time has passed for hybridization to be

DEPR:
Since three DPA equivalents bind per Ln ion, the preferred approach is to link the modified DPA to a polymeric lattice, which provides for close spacing of

chelators and can be designed to have useful DNA or RNA binding properties. These results suggest that a fused bicyclic DPA derivative is the candidate of choice.

DEPR:
FIG. 8 is a chemical drawing showing lanthanide chelators. The two classes of polymeric latice as illustrated in FIG. 8 can be employed for attachment of DPA derivatives, both based upon the use of synthetic polypeptides in the 10.sup.4 MW range. Synthesis can be conducted as described for simple DPA-peptide conjugates. The first polymer is to be used for covalent attachment to RNA via the transamination reaction to cytosine. This peptide lattice can be simple poly-L-lys. The second approach involves the coupling of modified DPA to a DNA binding peptide, which can be used to deliver the Ln chelate to RNA by means of non-covalent nucleic acid binding. For example, peptides can be synthesized in solution as a Lys.sub.3 Arg.sub.1 random co-polymer (average mw 10.sup.4). Subsequent to the conversion of Lys residues to the modified DPA conjugate, RNA binding can be driven by association with multiple Arg equivalents, taking advantage of the known helix selectivity of polyarginine. As for ethidium bromide (EB), addition of the non-covalent chelator conjugate can be made after washing to retain hybridization stringency.

DEPR:
An electronic schematic of the proximal detector/imager to be used with the multiplexed molecular analysis system is shown in FIG. 9. FIG. 9 is a diagram showing a multiplexed molecular analysis system electronics schematic. As illustrated in FIG. 9, the reaction vessel is placed directly on the fiber optic faceplate which is bonded to the sensor array. The faceplate provides sensor isolation to accommodate routine cleaning, as well as affording thermal isolation for ultrasensitive detection under cooled sensor operation. Also the optical faceplate can serve to filter excitation radiation by employing selective coatings. The sensor array is comprised of a plurality of smaller sensors such that the composite array approaches the surface area of the reaction vessel. The excitation source serves to excite the fluorescent reporter groups attached to the target molecules. Depending on the chosen reporter groups, the excitation source can be either a UV lamp, laser, or other commonly used light source used by those skilled in the art. The sensor array driver circuitry includes clocking, biasing and gating the pixel electrodes within the sensors. The cooling circuitry controls the thermoelectric cooler beneath the sensor array to enable ultrasensitive detection by providing very low thermal noise. Basically, the user selects the required temperature of operation and through feedback circuitry, the sensor array is held constant at such temperature. The image receive circuitry is responsible for obtaining the digital image from the sensor array and includes preamplification, amplification, analog to digital conversion, filtering, multiplexing, sampling and holding, and frame grabbing functions. Finally, the data processor processes the quantitative imaging data to provide the required parameters for the molecular analysis outcome. Also, a computer display is included for displaying the digital image.

DEPR:
The biosites are deposited by one of the several methods disclosed, either before or after the bottoms are bonded to the plate. In both situations, the probe molecules comprising the individual biosites must be attached to the glass or plastic surfaces.

DEPR:
In a preferred embodiment, thin (50-300 .mu.m) vinyl substrates are amino or epoxy functionalized with silanes similar to glass substrates. Thin vinyl substrates are immersed in a 1-2% aqueous solution of polyvinyl alcohol at 65.degree. C. The adsorbed polyvinyl alcohol is then reacted with either epoxy silane or amino silane, thus functionalizing the polymeric hydroxyl groups. Such optically clear vinyl substrates have the distinct advantage of blocking a large amount of the UV excitation source incident on the proximal CCD detector, but allowing the longer wavelengths (e.g. 500-650 nm) to pass through efficiently. This allows for greater sensitivity of labeled detector molecules that emit in

such wavelength region.

DEPR:
Nucleic acid probe attachment to glass employs well-known epoxy silane methods (see FIG. 12) described by Southern and others (U. Maskos el al., Nucleic Acids Res (1992) 20:1679-84; S. C. Case-Green et al, Nucleic Acids Res (1994) 22:131-36; and Z. Guo et al., Nucleic Acids Res (1994) 22:5456-65). FIG. 12 is a diagram showing glass and polypropylene surface coupling chemistries. With 3' amine-modified probes, covalent surface densities can be obtained having 10.sup.11 molecules/mm.sup.2 which is near the theoretical packing density limit. Amino-modified polypropylene is a convenient alternative to a glass substrate since it is inexpensive and optically clear above 300 nm. Amine-modified polypropylene can be converted to a carboxylic acid-modified surface by treatment with concentrated succinic anhydride in acetonitrile. Amine-modified probe is then coupled to this surface by standard carbodiimide chemistry in H.sub.2 O to yield probes at densities near 10.sup.9 /mm.sup.2 (see FIG. 12).

DEPR:
Similarly for probe-based diagnostics, target molecules derived from a patient sample can be dispensed into a single well containing numerous biosites for diagnosing genetic or infectious diseases. For example, single-stranded nucleic acid probes which are complementary to 96 known mutations of cystic fibrosis are arranged within a single well in a microplate. Upon hybridization with the patient's DNA sample, the resulting binding pattern obtained from the proximal CCD detector/imager indicates the presence of such known mutations.

DEPR:
in each reaction that had been extended by a single base are protected from digestion. All other DNA is degraded to mononucleotides. Following a brief thermal denaturation of the exonuclease, the contents of all the wells is robotically transferred to a new 384-well microtiter plate containing sequence complements microarrayed in a 5.times.5 microarray attached to the bottom of each well. Each of the 25 primers that had not been digested would hybridize to its corresponding complement in the array and imaged on the CCD detector to define the genotype at each loci.

DEPR:
FIG. 15 illustrates this homogenous multiplexed approach for the Polymerase Chain Reaction (PCR) simultaneously at 3 different loci. FIG. 15 is a diagram showing homogeneous in situ microarray detection of multiplexed PCR amplicons. FIG. 15 illustrates specific multiplex hybridization detection of PCR products using microtiter-based microarrays. Briefly, in this figure three separate amplification loci are being detected simultaneously. Each locus (e.g, PCR LOCUS 1) is defined by two specially modified amplification primers that define the ends of the amplified PCR product. One primer in the pair, contains a fluorescently detectable label such as fluorescein. The other primer in the pair contains two domains, one is a unique universal sequence complementary to a capture probe arrayed at the bottom of a single microtiter well and the other domain specific for template amplification. The universal sequence is attached to the amplification primer in a 5' to 5' linkage so that when the polymerase is amplifying the region of interest it does not jump over this specialized juncture, leaving the universal sequence as a single stranded motif. If a particular template in a sample well being amplified contains both primer loci (i.e., detection and capture sites), then a PCR product will be generated that can simultaneously hybridize and be detected to a complementary member of a universal capture array by the CCD proximal detector. Since only PCR amplicons hybridized to members of the universal array at the bottom of each well are proximal to the detector, the assay requires no special separation step to detect hybridized amplicons and thus becomes homogenous in nature.

DEPR:
Similarly, FIG. 16 illustrates this multiplexed concept with Gap-Ligase Chain Reaction (G-LCR). FIG. 16 is a diagram showing homogeneous in situ microarray detection of multiplexed gap-ligase chain reaction products. The ability to

detect hybridization events homogeneously is provided by the fact that only molecules proximally associated with specific biosites can be imaged by the detector. FIG. 16 illustrates specific multiplex hybridization detection of Gap-Ligase Chain Reaction products using microtiter-based microassays. Similarly, as described previously for PCR products (see FIG. 15), this figure illustrates the assay at three separate ligation-dependent amplification loci simultaneously. Each locus (e.g., LOCUS 1) is defined by two specially modified primers that define the ends of the gap ligase chain reaction product. One primer in the pair, contains a fluorescently detectable label such as fluorescein. The other primer in the pair contains two domains, one is a unique universal sequence complementary to a capture probe arrayed at the bottom of a single microtiter well and the other domain is specific for a region on the template being detected. The universal sequence attached to this primer serves as a sequence specific single stranded handle. When the template is present in the sample then sequence directed ligation will join both the label and the universal handle into a single product. After many cycles this amplified ligated product can be simultaneously hybridized and detected to its complementary member on a universal capture array immobilized to the bottom of a microtiter well and imaged by the CCD proximal detector. Since only ligated products hybridized to members of the universal array at the bottom of each well are proximal to the detector, the assay requires no special separation step to detect hybridized amplicons and thus becomes homogenous in nature.

DEPR:
Small molecule Universal Arrays are made by covalent attachment of small molecules such as those found in Table 1 to substrate surfaces. Immobilization of haptens, steroids, or drugs is accomplished by introducing a functionalized moiety at one end of the small molecule. These moieties are well known to those skilled in the art (e.g. N-hydroxy-succinimide, maleimide, isothiocyanate, iodoacetamide or other amine or sulfur reactive moieties). Small functionalized molecules or drugs can then be reacted with NH.sub.2 or SH.sub.2 derivitized plastic or glass substrates. Some specific examples of such commercially available activated haptens include NHS-fluorescein, NHS-biotin, NHS-digoxigenin, maleimide-biotin, and maleimide-tetramethylrhodamine.

DEPR:
Following deposition of the individual small molecule biosites, a bispecific ligand can be used to spatially localize specific binding events to given biosites. The bispecific ligand can comprise, but is not limited to, antibody-antibody conjugates, antibody-receptor, antibody-streptavidin, antibody-peptide, antibody-small molecule conjugates or bispecific antibodies.

DEPR:
The bispecific ligand is specific to both the immobilized hapten or drug on the substrate surface (biosite) and the analyte being screened. Examples of Universal Array screening are diagramed in FIG. 17. FIG. 17 is a diagram showing small molecule universal array (drug screening/discovery). FIG. 17 illustrates the basic small molecule Universal Array concept using four different immobilized haptens in a single well. Various bispecific molecules are diagramed for illustration purposes. FIG. 17 illustrates four separate and distinct haptens immobilized at the bottom of each of 96 wells of a microtiter plate. Each locus or biosite in the array is defined by four unique immobilized haptens illustrated in this example by fluorescein, digoxigenin, 2,4 dinitrophenol, and TRITC. Bispecific molecules uniquely specific for both the immobilized hapten and another labeled analyte in the sample are added to each well. In this fashion, different multiple analytes can be simultaneously detected and their presence indicated by signals at specific hapten biosites. In this example, 96 individual samples can be assayed for four different analytes simultaneously. As shown, the fluorescein biosite detects a labeled receptor (protein) analyte, both the 2,4 dinitrophenol and digoxigenin haptens allow for the simultaneous detection or presence of two additional types of protein receptors in the sample. Finally, the TRITC hapten allows for detection and presence of a specific enzyme substrate via an intervening enzyme conjugate. Once again, the proximal mode of detection allows for homogenous imaging of only those binding events at the surface of the array. The advantages of such a multiplexed

immunological approach is the exquisite specificity and variety of small molecules that comprise such a Universal Array using non-DNA based recognition of biosites.

DEPR:
The multiplexed molecular analysis system is also useful for analyzing the expression of hundreds of different mRNA species in a single tissue sample within a single well of a microtiter plate. Here synthetic nucleic acids form the distinct biosites which constitute numerous highly sensitive and selective hybridization analyses per sample, employing only 50 .mu.L of sample extract. Such massive hybridization analyses enables the discovery and employment of numerous biomarkers for specific diseases such as cancer. Essentially, the search for biomarkers of early phase lung cancer becomes an iterative, combinatorial process. For lung cancer and other epithelial disease, several hundred mRNAs are analyzed for their value as biomarkers at relatively low cost. In such an iterative process, the biostatistician becomes the end-user of the technology and a central component in the development of the final set of mRNA biomarkers. Once an mRNA biomarker set is discovered by this iterative approach, the technology is naturally suited for low cost, high throughput screening of large patient populations with the mRNA biomarker set of choice.

DEPR:
The procedure is to add a complex cellular mixture, e.g., a biological sample (for example, blood or sputum), to such an antibody matrix, then with some local mixing, allowing the cells to bind to the surface. If cells bind to such a matrix with good affinity and selectivity, they are then fixed to the matrix, permeabilized, and subjected to labeled probe hybridization (or PCR) in a fashion which is nearly identical to the methods which are currently used to analyze DNA or RNA in cells for microscopy or flow cytometry.

DEPR:
Microorganism monitoring applications can also be addressed by the multiplexed molecular analysis system. In particular for monitoring air, water, and food for microorganisms, the system can rapidly and cost effectively provide detection and quantification of complex biological mixtures. An example would be a ribosomal RNA probe-based assay in which nucleic acid probes serving as the biosites are chosen to selectively capture RNA of characteristic microorganisms.

DEPV:
Maskos, U., et al., Nucleic Acids Res. 20:1679-1684 (1992).

DEPV:
Stephen C. Case-Green, et al., Nucleic Acids Res. 22:131-136 (1994).

DEPV:
Guo, Z., et al., Nucleic Acids Res. 22:5456-5465 (1994).

DETL:
NUCLEIC ACID ANALYTE(TP2) CAPTURE PROBE(TP1) 5'-----CCACACTGGAACTGAGA------3'
5'-TGATTCAGACCGGCCG-3'a IIIIIIIIIIIIIIII IIIIIIIIIIIIIII
3'-GGTGTGACCTTGACTCT-----(Tn)---ACTAAGTCTGGCCGGC-5' TARGET PROBE 2 T LINKER
TARGET PROBE 1 - NUCLEIC ACID ANALYTE CAPTURE PROBE
5'-----CCACACTGGAACTGAGA------3' 5'-TGATTCAGACCGGCCG-3'a IIIIIIIIIIIIIIII
IIIIIIIIIIIIIII 3'-GGTGTGACCTTGACTCT-----(X)-----ACTAAGTCTGGCCGGC-5' TARGET
PROBE 2 X LINKER TARGET PROBE 1 - X = -OPO.sub.2 -{O-CH.sub.2 -CH.sub.2
-O-OPO.sub.2 -].sub.2 -O DIETHYLENE GLYCOLOATE LINKAGE - TARGET PROBE 1
3'AMINE----------5'-OPO.sub.2 -O-CH.sub.2 -CH.sub.2 -CH.sub.2 -CH.sub.2
-CH.sub.2 -CH.sub.2 -SH - 3' PROPYL AMINE TARGET PROBE 2 NH2-CH.sub.2
-CH(OH)-CH2-OPO.sub.2 -].sub.2 -O-3'----------5' - IODO ACETATE DERIVATIVE
TARGET PROBE 2 I-CH2-CO-NH-CH.sub.2 -CH(OH)-CH2-OPO.sub.2 -].sub.2
-O-3'----------5' - COUPLED TP1 + TP2 PRODUCT - 5' THIOL DERIVATIVE OF TP1 3'
ALKYL HALIDE OF TP2 - 3'-[TP1]-OPO.sub.2 -O-CH.sub.2 -CH.sub.2 -CH.sub.2
-CH.sub.2 -CH.sub.2 -CH.sub.2 -S-CH.sub.2 -CO-NH-CH.sub.2 -CH(OH)- CH.sub.2
-OPO.sub.2 -].sub.2 -O-[TP2]-5' - TP1 = target probe 1 TP2 = target probe 2

CLPR:
21. The device of claim 1, wherein said capillary tubes are free to flex between said attachment site and said array template.

CLPV:
an attachment site for holding said capillary tubes at a point spaced from the distal ends of such capillary tubes;

CLPV:
an array template for slidably holding each capillary tube near its distal end, and for allowing the distal end of each capillary tube to move with respect to the attachment site;

CLPV:
an attachment site for holding a plurality of flexible capillary tubes at a point spaced from the distal ends of such capillary tubes;